[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, January 1, 1932,
Vol. 55, No. 1, pp. 91–99]

THE TRANSFORMATION IN VITRO OF R PNEUMOCOCCI INTO S FORMS OF DIFFERENT SPECIFIC TYPES BY THE USE OF FILTERED PNEUMOCOCCUS EXTRACTS

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(Received for publication, October 17, 1931)

Griffith in 1928 (1) demonstrated conclusively for the first time that pneumococci of one specific type may, by suitable methods, be converted into pneumococci of heterologous types. He effected the change by injecting subcutaneously into mice small amounts of culture of living, non-virulent R forms together with large amounts of heat-killed S pneumococci of a type other than that of the organisms from which the R cells were derived. Living virulent S organisms were recovered from heart's blood cultures of the animals. The type of the S organisms recovered was not that of the pneumococci from which the R organisms had been derived, but was that of the heat-killed S forms employed in the experiment. Hence a definite transformation in type had occurred. Rigid controls excluded the possibility of S forms being contained in the heat-killed suspensions.

Neufeld and Levinthal (2) and Dawson (3), using similar technic, confirmed Griffith's findings. All of these writers were unable to bring about a change of type *in vitro*. In a subsequent paper, however, Dawson and Sia (4) reported experiments in which it had been possible to effect a transformation of type *in vitro*. Their method was to inoculate small quantities of R pneumococci derived from S organisms of one type into blood broth containing anti-R serum and the heat-killed S pneumococci of another type. When solutions of S organisms broken up by freezing and thawing and subsequently heated to 60°C. were substituted for the whole bacteria, no transformation occurred.

In the studies just described, transformation of pneumococci of one specific type into those of another type was accomplished only by adding to living R cultures the whole heat-killed bodies of S pneumococci. In attempting to analyze the nature of this phenomenon it seemed desirable to determine whether the active principle responsible for the transformation could be extracted in soluble form from the S cells.

In the present paper experiments are reported on the use of cell-free,

heated and filtered extracts of S pneumococci in inducing conversion of R forms to the same specific type of Pneumococcus as that of the organisms from which the extract was prepared.

EXPERIMENTAL

Methods

Cultures—Cultures of R pneumococci used in the experiments were stock strains originally derived from type-specific S pneumococci by growth in broth containing 10 per cent homologous immune serum. The organisms from which the extracts were prepared were type-specific strains of S pneumococci the virulence of which had been maintained by frequent animal passages.

Preparation of Pneumococcus Extract.—Cultures of S pneumococci designed for extraction were grown in meat infusion broth, pH 7.8, containing dextrose 0.01 per cent. Just prior to inoculation the medium was boiled for 10 minutes, then cooled rapidly to a temperature suitable for growth. Cultures were grown under a seal of vaseline or liquid paraffin for 10 or 12 hours, depending on the density of growth.

Organisms from 5 l. of broth culture of S pneumococci so prepared were thrown down by centrifugation and were collected in a volume of approximately 40 cc. of sterile distilled water. The suspension was placed in a heavy pyrex tube and immediately covered with sterile liquid paraffin. The concentrated suspension was then frozen and thawed rapidly until the organisms were broken up, seven or eight freezings usually being necessary. Dry ice in alcohol was used to freeze the mixture. The preparation was immediately placed in a water bath and heated to 60°C. for 30 minutes, following which it was centrifuged at high speed for 2½ hours. The supernatant fluid was diluted with sterile distilled water to a volume of 500 cc., made alkaline by the addition of 1.0 cc. of normal sodium hydroxide, and passed through a Berkefeld N filter. The filtrate was partially neutralized by the addition of 0.7 cc. of normal hydrochloric acid. Then, by vacuum distillation, at low temperature, the solution was concentrated to a volume of 50 cc., that is to 1/100 the volume of the original broth culture. The extract was finally heated in a water bath to 60°C. for 10 minutes to insure sterility. All extracts were tested for sterility by culture in broth and by animal inoculation. In no instance were viable pneumococci isolated from the heated filtrates.

Anti-R Serum.—The anti-R serum used originally was prepared by the intravenous injection into rabbits of suspensions of heat-killed R pneumococci. It was subsequently found that normal hog serum[1] served equally well. Since hog serum

[1] Recent studies of Sia (5) have shown that normal hog serum has definite antagonistic properties against S forms of pneumococci. These findings have been confirmed by Kelley in this laboratory who found also (6) that the anti-R titre of normal hog serum is frequently as high as 1/512, the average being slightly lower.

was more easily obtained in large quantities it was substituted for anti-R rabbit serum.

Cultural Technic.—The *in vitro* experiments of Dawson and Sia (4) showed that the size of the inoculum of the R cells exerts a determining influence on the result. Small inocula are essential for transformation. The present experiment was prepared as follows: One drop of an R culture prepared by diluting 0.1 cc. of an 8 hour culture in 5 cc. of sterile broth, was placed in a tube containing 1.5 cc. of broth, 1.0 cc. of the filtered extract, and 0.3 cc. of hog serum. The mixture was immediately covered with a thick layer of sterile liquid paraffin and incubated at 37°C. Transfers were made serially every 24 hours, 1 drop of culture being carried over to each tube of the second series. No experiment was considered negative until at least five subcultures had been made. As subcultures were prepared, transfers were also made to blood agar plates, so that the characteristics of the colonies might be studied. Whenever smooth colonies were noted, one such colony was picked and transferred to blood broth. The organisms from the cultures were subsequently typed and tested for virulence in mice.

Conversion of an R Strain Derived from Type II Pneumococci into a Type III S Strain

Since the filtered extracts were found to vary considerably in concentration and there was variation also in the behavior of cultures, even when grown in the same lot of extract, it was found advisable to set up several tubes for each experiment. The protocol and results of a characteristic experiment are given in Table I.

From Table I it can be seen that when an R strain of Pneumococcus derived from Type II S organisms was grown in broth containing a filtered extract of Type III S organisms and a small amount of anti-R serum, viable S pneumococci were recovered after 24 hours which were specifically agglutinable in Type III antiserum. They were encapsulated, formed large flat mucoid colonies on blood agar, and exhibited all the characteristics of Type III S pneumococci. Moreover, these organisms in the first culture after isolation were highly virulent for mice as is shown in Table II.

The acquisition of virulence seems rather remarkable in view of the fact that the R strain of Pneumococcus prior to modification was completely avirulent. Inocula of 0.75 cc. of the original R strain injected intraperitoneally into mice did not cause death, and on many occasions when the organisms from 15 cc. of culture were injected subcutaneously the animals lived. After transformation the newly

TABLE I

Conversion of Strain of R Pneumococcus Derived from Type II S into Type III S by Means of a Filtered Extract of Type III S Pneumococci

Tube	Amount of broth	Strain of R pneumococcus	Filtered extract of Type III S pneumococci	Anti-R serum (normal hog)	Type of colonies*	Specific agglutinability of S colonies
	cc.		*cc.*	*cc.*		
1	1.5	D 39 R**	1.0	0.3	R and S	Type III
2	1.5	D 39 R	1.0	0.3	R and S	Type III
3	1.5	D 39 R	1.0	0.3	R and S	Type III
4	1.5	D 39 R	1.0	0.3	R and S	Type III
5	1.5	D 39 R	—	0.3	R only	—
6	1.5	—	1.0	0.3	Sterile	—

* Determined by plating on blood agar after 24 hours growth.

** R strain derived from Type II S pneumococci by growth in homologous immune serum broth.

TABLE II

Virulence Test in Mice of Pneumococcus Type III Derived from an R Strain (D 39 R) by Use of a Filtered Extract of Type III S Pneumococci*

Mouse	Amount of culture**	Result
	cc.	
1	0.1	D in 24 hrs.
2	0.01	D in 26 hrs.
3	0.001	D in 24 hrs.
4	0.0001	D in 26 hrs.
5	0.00001	D in 60 hrs.
6	0.000001	D in 5 days
7	0.0000001†	S 7 days

* R strain derived from Type II S pneumococcus by growth in homologous immune serum broth.

** Type III S pneumococcus derived from D 39 R by means of filtered extract of Type III S.

† This inoculum seeded into agar yielded no growth.

D = died.

S = survived.

derived Type III culture proved fatal to mice in amounts as small as 0.000,001 cc.

Conversion of an R Strain Derived from Type II Pneumococci into a Type I S Strain

It seemed of interest, in view of the fact that an R strain derived from Type II organisms could be transformed through the use of an

TABLE III

Conversion of Strain of R Pneumococcus Derived from Type II S into Type I S by Means of a Filtered Extract of Type I S Pneumococci

Tube	Amount of broth	Strain of R pneumococcus	Filtered extract of Type I S pneumococci	Anti-R serum (normal hog)	Type of colonies	Specific agglutinability of S colonies
			Original culture			
	cc.		*cc.*	*cc.*		
1 *a*	1.5	D 39 R*	1.0	0.3	R only	—
2 *a*	1.5	D 39 R	1.0	0.3	R only	—
3 *a*	1.5	D 39 R	1.0	0.3	R only	—
4 *a*	1.5	D 39 R	1.0	0.3	R only	—
5 *a*	1.5	D 39 R	—	0.3	R only	—
6 *a*	1.5	—	1.0	0.3	Sterile	—
			First subculture			
1 *b*	1.5	From Tube 1 *a*	1.0	0.3	R only	—
2 *b*	1.5	From Tube 2 *a*	1.0	0.3	R only	—
3 *b*	1.5	From Tube 3 *a*	1.0	0.3	R only	—
4 *b*	1.5	From Tube 4 *a*	1.0	0.3	R only	—
5 *b*	1.5	From Tube 5 *a*	—	0.3	R only	—
			Second subculture			
1 *c*	1.5	From Tube 1 *b*	1.0	0.3	R and S	Type I
2 *c*	1.5	From Tube 2 *b*	1.0	0.3	R and S	Type I
3 *c*	1.5	From Tube 3 *b*	1.0	0.3	R and S	Type I
4 *c*	1.5	From Tube 4 *b*	1.0	0.3	R and S	Type I
5 *c*	1.5	From Tube 5 *b*	—	0.3	R only	—

* D 39 R = an R strain derived from Type II S pneumococci by growth in homologous immune serum.

extract from Type III cells into typical Type III pneumococci, to determine whether these same R forms might be changed into Type I pneumococci by means of a Type I extract. Such an experiment is recorded in Table III. It will be noted that in this instance no trans-

formation in type occurred until the second subculture in the extract medium had been made.

From Table III it can be seen that after an R strain of Pneumococcus derived from Type II S pneumococci was passed through three transfers in broth containing anti-R serum and a filtered extract of Type I culture, organisms were recovered which showed the characteristics of Type I pneumococci. They formed smooth colonies on blood agar, agglutinated specifically in Type I antiserum, possessed capsules, and were virulent for mice.

The conversion of R forms into organisms of Type I has been consistently more difficult than their transformation into Type III pneumococci. In the former instance three, four, and even five subcultures in the extract medium have been necessary.

DISCUSSION

The conditions affecting the activity of the filtered extracts are on the whole similar to those influencing the potency of the bacterial suspensions used by Griffith and by Dawson in their experiments. Autolysis of cells during the preparation of the extract must be minimized to avoid loss of activity. Aging of the extract tends to diminish its potency. Growth under anaerobic conditions facilitates but is not essential for the transformation.

The nature of the rôle of anti-R serum in the conversion is not fully understood. It is known (7) that growth of R cultures in broth containing anti-R serum usually results in their reversion to S organisms of the specific type from which they were originally derived. It has been found that transformations occur when normal sheep, rabbit, guinea pig, horse, or human serum is employed. These sera differ markedly in their anti-R titre and the observations so far seem to indicate that some other property of these sera may be the one concerned in the reaction. Thus far it has not been possible entirely to dispense with serum in the culture medium.

Despite the fact that the capsular polysaccharide of the Pneumococcus determines its type specificity, this substance alone, when added in chemically purified form, has not been found effective in causing transformation of R organisms derived from pneumococci of one type into S forms of other types. Numerous experiments in

which specific polysaccharides were used in varying dilutions together with anti-R serum and R cultures yielded only negative results.

It is believed that the procedures carried out in the preparation of the filtered and heated extracts exclude the possibility of any viable pneumococci being carried over in the extracts. It seems very unlikely, despite recent reports on filtrable forms of bacteria, that any living element can persist after the heating and filtration to which the material is subjected. Careful controls of sterility, including frequent serial transfers and mouse inoculation, have been invariably negative. Consequently the appearance in R cultures grown in the extract medium, of S forms belonging to the same specific type as that of the extracted pneumococci, is believed to indicate that the R cells originally derived from one type of Pneumococcus have acquired the characteristics of S forms of a different type. Once the organisms have assumed the specific characters, they remain true to form through repeated transfers in ordinary media and through repeated animal passages.

Filtered extracts prepared as described are not invariably active. Several preparations have failed to effect a change despite repeated subcultures in the extract-containing medium. Moreover, out of a group of six tubes identically prepared, so far as can be determined, two or three may give positive results while the others remain negative. Although attempts to convert R pneumococci derived from Type I or Type III strains into S organisms of other types through the use of extracts have thus far been unsuccessful, it seems likely that by proper modification of the technic, these changes may be accomplished. Marked differences are encountered in the ease with which R organisms may be transformed even when suspensions of heat-killed cells are used. In view of the additional manipulation incident to the preparation of extracts, and the probable loss of the specific principle responsible for the change, it is not surprising that failures are encountered when such extracts are employed.

When R pneumococci change into the S forms they always acquire the property of producing the specific capsular substance. The immunological specificity of the encapsulated cell depends upon the chemical constitution of the particular polysaccharide in the capsule. The synthesis of this specific polysaccharide is a function peculiar to

S forms of pneumococci. However, since the R cells under suitable conditions have been found to develop again the capacity of elaborating this specific material, it appears that in them this function is potentially present, but that it remains latent until activated by special environmental conditions. The fact that an R strain derived from one type of Pneumococcus, under the conditions defined in this paper, may be caused to acquire the specific characters of the S form of a type other than that from which it was originally derived implies that the activating stimulus is of a specific nature.

Since this paper was submitted for publication, two articles by Dawson and Sia (8, 9) have appeared on the transformation of pneumococcal types *in vitro*. In the second of the two papers the authors describe experiments in which they were unable to effect a transformation of type *in vitro* by using extracts made under anaerobic conditions by freezing and thawing 20 or more times suspensions of type-specific pneumococci. In a personal communication, Dawson states he has found recently that suspensions of S pneumococci which are frozen and thawed less than eight times are still active in inducing transformation in type.

CONCLUSIONS

1. Avirulent R pneumococci derived from S forms of a specific type may be changed by growth in broth containing anti-R serum and a heated, filtered extract of S pneumococci of a different type, into virulent S organisms identical in type with the bacteria extracted. This has been accomplished in the case of R strains derived from Type II pneumococci, employing extracts prepared from Type III and Type I S forms.

2. The constituents of the extract supply an activating stimulus of a specific nature in that the R pneumococci acquire the capacity of elaborating the capsular material peculiar to the organisms extracted.

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